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ORIGINAL ARTICLE

Clinical significance of CADM1/TSLC1/IgSF4 expression in adult T-cell leukemia/lymphoma

S Nakahata[1], Y Saito[1], K Marutsuka[2], T Hidaka[3], K Maeda[3,4], K Hatakeyama[5], T Shiraga[1,6], A Goto[1], N Takamatsu[1], Y Asada[5], A Utsunomiya[7], A Okayama[8], Y Kubuki[3], K Shimoda[3], Y Ukai[9], G Kurosawa[9] and K Morishita[1]

Cell adhesion molecule 1 (CADM1/TSLC1) was recently identified as a novel cell surface marker for adult T-cell leukemia/lymphoma (ATLL). In this study, we developed various antibodies as diagnostic tools to identify CADM1-positive ATLL leukemia cells. In flow cytometric analysis, the percentages of $CD4^+CADM1^+$ double-positive cells correlated well with both the percentages of $CD4^+CD25^+$ cells and with abnormal lymphocytes in the peripheral blood of patients with various types of ATLL. Moreover, the degree of $CD4^+CADM1^+$ cells over 1% significantly correlated with the copy number of the human T-lymphotropic virus type 1 (HTLV-1) provirus in the peripheral blood of HTLV-1 carriers and ATLL patients. We also identified a soluble form of CADM1 in the peripheral blood of ATLL patients, and the expression levels of this form were correlated with the levels of soluble interleukin 2 receptor alpha. Moreover, lymphomas derived from ATLL were strongly and specifically stained with a CADM1 antibody. Thus, detection of $CD4^+CADM1^+$ cells in the peripheral blood, measurement of serum levels of soluble CADM1 and immunohistochemical detection of CADM1 in lymphomas would be a useful set of markers for disease progression in ATLL and may aid in both the early diagnosis and measurement of treatment efficacy for ATLL.

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Keywords: CADM1/IgSF4/TSLC1; ATLL

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) results from infection with human T-lymphotropic virus type 1 (HTLV-1).[1,2] Following HTLV-1 infection, 2.1 to 6.6% of HTLV-1 carriers will develop ATLL, and most of the ATLL patients will die within a year.[3] An estimated 10-20 million people worldwide are infected with HTLV-1, and HTLV-1 is endemic in southwestern Japan, the island of Kyushu, Africa, the Caribbean Islands and South America.[4] ATLL cells are mainly derived from activated helper T cells with the $CD3^+$, $CD4^+$, $CD8^-$ and $CD25^+$ (also known as interleukin 2 receptor alpha (IL-2Rα)) cell surface markers.[2] A fraction of ATLL cases have been shown to also express forkhead box P3 (*FOXP3*), which is a master gene for regulatory T cells (T-reg), suggesting that some cases of ATLL may originate from HTLV-1-infected T-reg cells.[5,6] For diagnosis, identification of mono- or oligoclonal provirus integration events by Southern blot analysis is one of the definitive markers for ATLL. In addition to viral integration, ATLL cells with multi-lobulated nuclei (called 'flower cells') have been frequently seen in leukemia cells in the peripheral blood of ATLL patients. Hypercalcemia and high levels of either serum lactate dehydrogenase (LDH) or soluble IL-2Rα (sIL-2Rα) have been found to be unfavorable markers for ATLL; however, these markers are not specific for the diagnosis of ATLL.[7,8]

The developmental steps of ATLL after HTLV-1 infection have remained obscure for 30-40 years. HTLV-1 Tax is thought to be an important viral protein that functions in the maintenance of HTLV-1-infected lymphocytes;[9,10] however, expression of Tax protein was not detected in over 70% of ATLL cases because of genomic deletion and/or DNA methylation.[11-14] Recently, HTLV-1 basic leucine zipper (HBZ) was found to be constitutively expressed in ATLL cells and was shown to interact with JUN and CREB2 to regulate Tax expression.[15,16] HBZ also promotes $CD4^+$ T-cell proliferation in transgenic mice;[16] therefore, HBZ has important roles and functions not only in maintaining the virus life cycle but also in the maintenance of the HTLV-1-infected cells that contribute to disease pathogenesis. Although HBZ is expressed in the majority of ATLL cells, only 5% of HTLV-1 carriers develop ATLL, suggesting that additional factors besides viral infection are required for the development of ATLL.

To identify additional pathogenic factors or novel surface markers for ATLL, we collected gene expression profiles for acute-type ATLL. Using a comprehensive DNA microarray gene expression analysis, we recently demonstrated that cell adhesion molecule 1 (CADM1/TSLC1/IgSF4) is a novel cell surface maker for ATLL.[17] CADM1 was initially isolated as a tumor suppressor for lung cancers by genomic analysis. CADM1 expression is reduced in a variety of cancers by promoter methylation and is associated with poor prognosis and enhanced metastatic potential.[18] By contrast, we identified that high expression of CADM1 has an important role in enhanced cell-cell adhesion to the vascular endothelium, tumor growth and the organ infiltration of ATLL cells.[19]

In this study, we developed various antibodies for CADM1 to be used as diagnostic tools for identifying ATLL leukemia cells.

[1]Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; [2]Pathology Division, University of Miyazaki Hospital, Miyazaki, Japan; [3]Department of Gastroenterology and Hematology, Faculty of Medicine, Miyazaki University, Miyazaki, Japan; [4]Department of Internal Medicine, Miyakonojo National Hospital, Miyazaki, Japan; [5]Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; [6]Department of Foods and Human Nutrition, Faculty of Human Life Sciences, Notre Dame Seishin University, Okayama, Japan; [7]Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan; [8]Department of Rheumatology, Infectious Diseases and Laboratory Medicine, University of Miyazaki, Miyazaki, Japan and [9]Division of Antibody Project, Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan. Correspondence: Professor K Morishita, Division of Tumor and Cellular Biochemistry, Department of Medical Science, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan.
E-mail: kmorishi@med.miyazaki-u.ac.jp
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We successfully identified ATLL cells in the peripheral blood and in lymphoma samples and detected the soluble form of CADM1 in the peripheral blood of ATLL patients using specific antibodies for CADM1. The CADM1 antibody may therefore represent a useful tool in the diagnosis of ATLL cells.

MATERIALS AND METHODS

Quantification of HTLV-1 proviral load

HTLV-1 proviral DNA load was determined by real-time PCR as previously described.[20] Briefly, genomic DNA from peripheral blood mononuclear cells (PBMCs) was extracted by proteinase K digestion and phenol/chloroform extraction and then subjected to a real-time TaqMan PCR assay using an ABI PRISM 7000 detection system (Perkin Elmer/Applied Biosystems, Forster City, CA, USA) with two sets of primers specific for the *pX* region of the HTLV-1 provirus and the human gene encoding the RNase P enzyme. The primers and the probe for the *RNase P* gene were purchased from Applied Biosystems; those for the *pX* region of the HTLV-1 provirus were described previously.[20] Genomic DNA of normal control PBMCs mixed with a plasmid DNA, which contained almost the whole genome of the HTLV-1 provirus (*Sac*I site of 5′-LTR to *Sac*I site of 3′-LTR), was used as a standard to quantify the proviral DNA copies. The copy number of the plasmid DNA was calculated based on the size and weight of the plasmid DNA, as measured by spectrophotometry. HTLV-1 proviral loads in some of the PBMC samples were measured by the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously.[21] The amount of HTLV-1 proviral DNA was calculated as the copy number of HTLV-1 per 100 PBMC = ((copy number of *pX*)/(copy number of *RNase P*/2)) × 100.

RESULTS

Frequent expression of surface CADM1/TSLC1 among ATLL-derived cell lines

CADM1/TSLC1/IgSF4 was identified as a novel surface marker on ATLL cells by gene expression profiling using DNA microarray analysis and was found to be frequently expressed in leukemia cells from patients with acute-type ATLL.[17] We first analyzed the CADM1 protein levels in a panel of T-leukemia cell lines using a chicken anti-human CADM1 antibody (MBL, Nagoya, Japan). A 107 kDa band was clearly detected in whole-cell lysates from the KOB, KK1 and S1T cell lines (Figure 1a), which have been reported

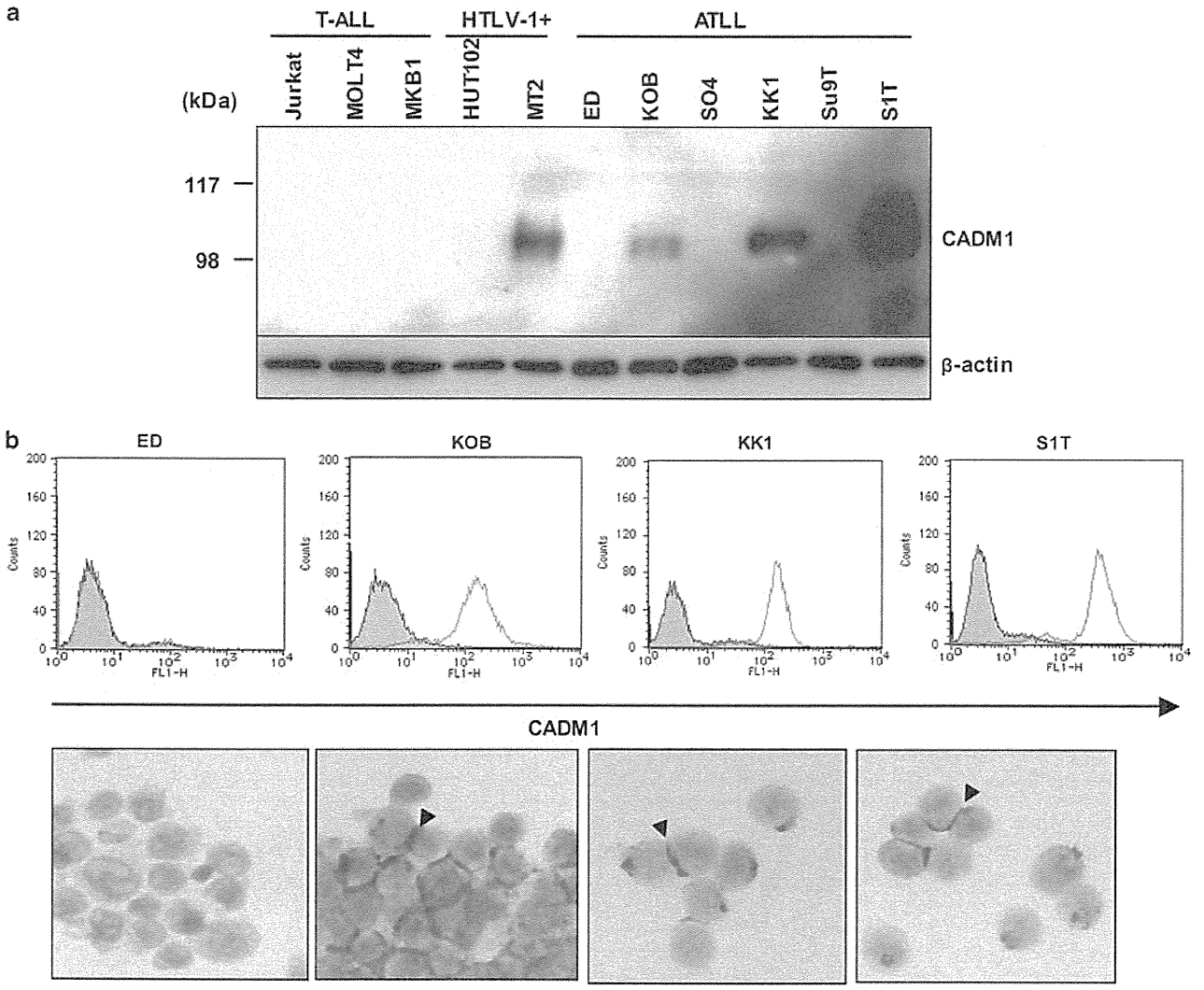

Figure 1. High CADM1 expression in ATLL analyzed by immunoblot, flow cytometry (FMC) and immunohistochemical staining (IHC). (**a**) Immunoblot analysis was performed on a series of T-lymphoid leukemia cell lines (three T-ALL, T-acute lymphoid leukemias; two HTLV-1 +, HTLV-1-infected cell lines; six ATLL, ATLL-derived cell lines) with a chicken anti-human CADM1 antibody. (**b**) A human anti-human CADM1 antibody (051–054), which was established by phage display, was used for FMC and IHC. The anti-CADM1 antibody was visualized by Alexa 488 in FMC and by horseradish peroxidase in IHC.

to express CADM1 according to reverse transcriptase PCR and northern blot analysis.[17] To confirm CADM1 expression on the cell surface of ATLL cells, we examined CADM1 membrane expression by flow cytometry with an Alexa 488-labeled human anti-CADM1 antibody generated by phage-display technology.[22] Four ATLL cell lines were used for flow cytometry: CADM1-negative ED and CADM1-positive KOB, KK1 and S1T cell lines. In all three CADM1-positive cell lines, the fluorescence intensity of CADM1 expression was two logs greater than that of the isotype immunoglobulin G control (Figure 1b, upper panels), while only background levels of fluorescence could be seen in the CADM1-negative ED-ATLL cell line, which had high levels of DNA methylation in the CADM1 promoter region.[17] To evaluate the subcellular distribution of CADM1, immunohistochemical staining was performed on the same cell lines using the anti-CADM1 antibody (Figure 1b, bottom panels). CADM1 was highly concentrated at the cell–cell contact sites in the three CADM1-positive cell lines, and no staining of CADM1 was detected in the ED cell line. These data suggest that CADM1 expression in ATLL cells may promote cell-to-cell contact.

Low levels of CADM1 expression in the T-reg fraction of peripheral lymphocytes

To examine the expression of CADM1 in peripheral blood T-lymphocytes of healthy volunteers, T-reg populations were analyzed for CADM1 expression because $CD4^+CD25^{high}$ T-reg cells are a potential source of ATLL cells.[5,6] Initially, the $CD4^+CD25^+$ cell fraction was separated from PBMCs of a healthy volunteer by the magnetic bead method and stained with an anti-CADM1 antibody. Almost 100% of the S1T-ATLL cell line was strongly stained with the anti-CADM1 antibody; however, 55.8% of the $CD4^+CD25^+$ cells were stained weakly in comparison with the high level of staining of S1T-ATLL cells (Figure 2a). To confirm whether the purified $CD4^+CD25^+$ cells expressing CADM1 were T-reg cells, the sorted $CD4^+CD25^+$ cells were stained for both FoxP3 (a master regulator in the development of T-reg cells) and CADM1. In all, 93% of the $CD4^+CD25^+$ double-positive cells in the peripheral blood were stained by the anti-FoxP3 antibody, while 37% of the cells were stained with both the anti-CADM1 and anti-FoxP3 antibodies (Figure 2b), suggesting that a fraction of the $CD4^+CD25^+FoxP3^+$ T-reg cells weakly expressed CADM1 on their cell surfaces.

We then determined the proportion of $CD4^+CADM1^+$ and $CD4^+CD25^+$ T cells in PBMCs from 10 healthy volunteers after selection with Cy5-labeled CD45 staining. On average, 7.3% of $CD45^+$ cells in PBMCs expressed CD4 and CD25, while only 0.6% of the cell population expressed CD4 and CADM1 (Figure 2c and representative fluorescence-activated cell sorting data are shown in Supplementary Figures 1a and b), indicating that the number of $CD4^+CADM1^+$ cells was significantly lower than the number of $CD4^+CD25^+$ cells in the PBMCs of healthy volunteers. To determine the percentage of $CD4^+CADM1^+$ cells in peripheral lymphocytes of various types of ATLL and HTLV-1 carriers, $CD45^+$ PBMCs from 40 patients diagnosed with various types of ATLL (7 acute-type, 4 lymphoma-type, 6 chronic-type and 23 smoldering-type), 51 HTLV-1 carriers and 10 normal volunteers were analyzed for the surface expression of CD4 and CADM1 by flow cytometry analysis, which was performed by double staining of CD12/CD19, CD3/CD8, CD4/CD25, CD23/CD5, CADM1/CD4, CD20/CD11c, CD16/CD56, CD30/CD7 and κ-chain/λ-chain. The median percentages of $CD4^+CADM1^+$ cells were 73.9% in acute cases, 72.4% in chronic cases (except for a patient with CD4-negative ATLL described below), 5.6% in lymphoma cases, 11.5% in smoldering cases, 4.4% in HTLV-1 carriers and 0.5% in normal volunteers (Figure 2d). In these subjects, the percentages of $CD4^+CD25^+$ cells were significantly correlated with those of $CD4^+CADM1^+$ cells ($R = 0.907$, $P < 0.0001$) (Figure 2e), suggesting that most of the ATLL cells were $CD4^+CD25^+CADM^+$. However, we also observed a cell surface profile of $CD3^+CD8^-$ (91.3%), $CD25^+CD4^-$ (81.5%) and $CD4^-CADM1^+$ (83.6%) in a case of chronic ATLL, suggesting that the surface markers of the ATLL cells represented $CD4^-CD8^-$ double-negative T lymphocytes that expressed CD25 and CADM1.

CADM1 expression in leukemia cells from ATLL patients and HTLV-1-infected cells from HTLV-1 carriers

To confirm that most of the HTLV-1-infected ATLL cells were indeed in the $CD4^+CADM1^+$ cell fraction, PBMCs from an HTLV-1 carrier and two ATLL patients with chronic or smoldering ATLL were isolated and separated into CADM1-positive and CADM1-negative cell fractions by anti-CADM1 antibody-conjugated magnetic beads. The cell fractions were then analyzed for the expression of CD4 and CADM1 by fluorescence-activated cell sorting analysis (Supplementary Figure 2). In these three patients, 3.4 to 31.4% of PBMCs were positive for CD4 and CADM1. After separation by the magnetic CADM1 antibody, 73.5 to 96.5% of the cells were $CD4^+CADM1^+$. To assess whether these $CD4^+CADM1^+$ cells indeed represented the HTLV-1-infected cell population, the HTLV-1 status was determined by PCR of the proviral DNA with primers against the *HBZ* region of the HTLV-1 genome. As shown in Figure 3a, the HTLV-1 genomic sequence was detected in the three CADM1-positive cell fractions, while weak or no signal was detected in the CADM1-negative cell fractions, indicating that the majority of HTLV-1-positive cells are present in the CADM1-positive cell fractions.

Next, the percentages of $CD4^+CADM1^+$ cells were compared with those of abnormal lymphocytes or with the DNA copy numbers of HTLV-1 in PBMCs of patients with various types of ATLL, which included 6 acute-type, 8 chronic-type and 6 smoldering-type of ATLL, and 20 HTLV-1 carriers (Figures 3b and c). The percentages of $CD4^+CADM1^+$ cells showed a high degree of correlation with those of abnormal lymphocytes ($R = 0.791$, $P < 0.0001$) and with the HTLV-1 DNA copy numbers ($R = 0.677$, $P < 0.0001$) in these patient samples. Notably, in two samples from chronic- and smoldering-type ATLL patients, the number of $CD4^+CADM1^+$ cells was less than one-half of the number of HTLV-1 DNA copies (32.0% vs 107.97 copies and 30.0% vs 65.76 copies), which may be due to multiple copies of proviral DNA in the cells. In addition, the percentages of $CD4^+CADM1^+$ cells were correlated with the levels of sIL-2Rα ($R = 0.586$, $P < 0.0001$) and with the levels of LDH ($R = 0.486$, $P = 0.0015$) (Figures 3d and e). Consistent with earlier studies, both serum sIL-2Rα and LDH levels were correlated with the HTLV-1 DNA copy numbers ($R = 0.705$; $P < 0.0001$ and $R = 0.44$; $P = 0.0045$, respectively) in this study (data not shown).

To further evaluate the diagnostic efficacy of measuring CADM1-positive cells to detect HTLV-1-infected cells, the copy number of the HTLV-1 provirus in PBMCs of carriers was compared with the percentages of $CD4^+CADM1^+$ cells and the serum levels of sIL-2Rα and LDH. The percentage of $CD4^+CADM1^+$ cells showed a significant correlation with the HTLV-1 DNA copy number ($R = 0.921$, $P < 0.0001$) (Figure 3f), while there was a poor correlation between HTLV-1 copy number and the levels of sIL-2Rα and LDH (data not shown). A correlation between the percentage of $CD4^+CADM1^+$ cells and abnormal lymphocytes was also observed in the HTLV-1 carriers ($R = 0.819$, $P < 0.0001$), although abnormal lymphocytes and $CD4^+CADM1^+$ cells were very rare in these subjects (Supplementary Figure 3). On the basis of these data, in addition to the determination of copy numbers of HTLV-1 proviral DNA, quantification of $CD4^+CADM1^+$ cell number by flow cytometry may be useful for monitoring the number of HTLV-1-infected cells in the peripheral blood of ATLL patients and HTLV-1 carriers.

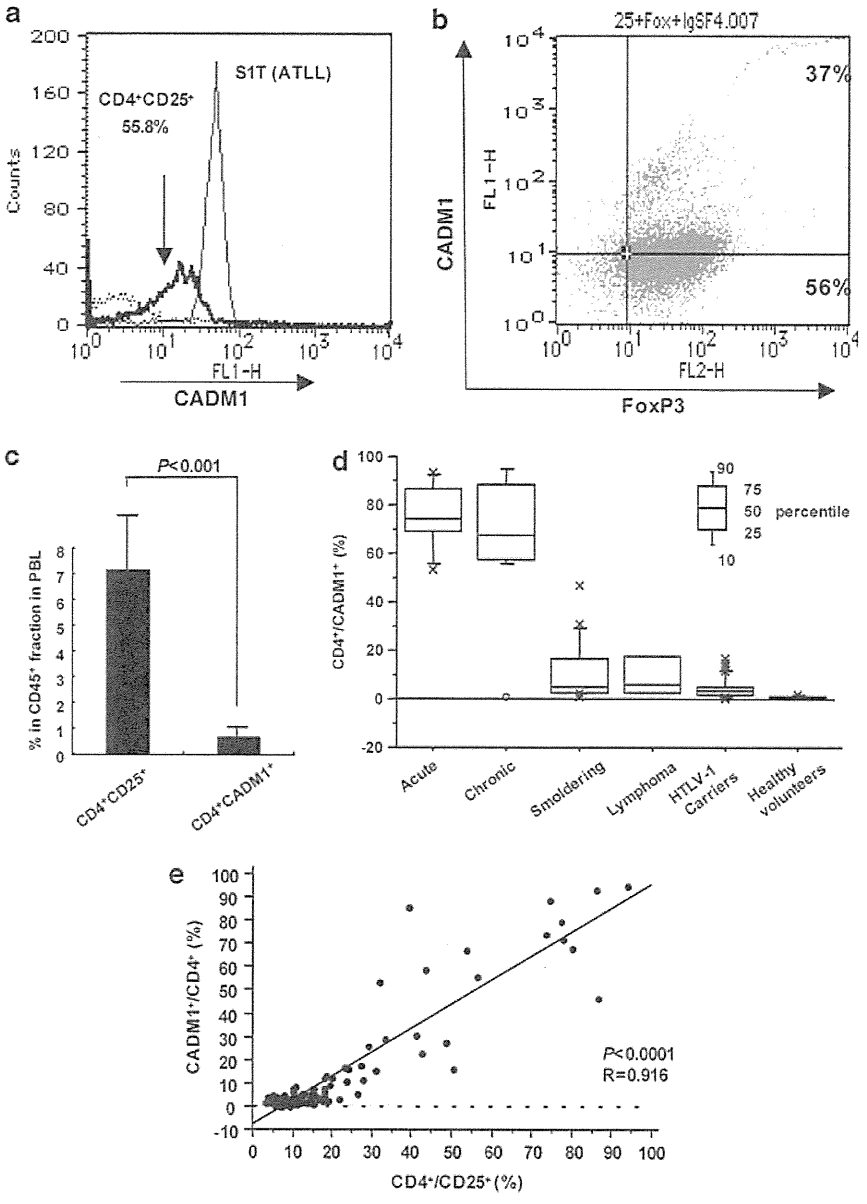

Figure 2. Flow cytometric analysis of CADM1 in T-reg lymphocytes, ATLL cells and HTLV-1-infected T cells. (**a**) Flow cytometric analysis of CADM1 expression in the $CD4^+CD25^+$ fraction from peripheral T lymphocytes. Each sample was stained with an Alexa 488-labeled anti-CADM1 antibody. The S1T-ATLL cell line with high CADM1 expression was used as a positive control. (**b**) The $CD4^+CD25^+$ fraction from peripheral lymphocytes was stained by the Alexa 488-labeled anti-CADM1 and PE-labeled anti-FoxP3 antibodies. (**c**) Comparison of percentages between the $CD4^+CD25^+$ and $CD4^+CADM1^+$ cell fractions in the $CD45^+$ fraction of peripheral blood lymphocytes. (**d**) Box plots are shown for the percentages of the $CD4^+CADM1^+$ cell fractions in $CD45^+$ peripheral blood lymphocytes from patients with various types of ATLL, HTLV-1 carriers and healthy volunteers. The data from a CD4-negative ATLL case are indicated by a white circle. (**e**) Comparison between $CD4^+CADM1^+$ and $CD4^+CD25^+$ cell fractions in $CD45^+$ peripheral blood lymphocytes from patients with various types of ATLL, HTLV-1 carriers and healthy volunteers. Spearman correlation coefficients were calculated to assess the association between $CD4^+CADM1^+$ and $CD4^+CD25^+$ cell fractions.

The soluble form of CADM1 is detected in the serum of ATLL patients

A soluble isoform of CADM1 consisting of the extracellular domain was recently isolated in murine mast cells.[23] We determined whether the soluble form of CADM1 was present in the serum of ATLL patients by western blot using a chicken anti-human CADM1 antibody. As a positive control, soluble CADM1 was produced by transfection of 293 cells with a construct encoding a soluble form of CADM1 (1 to 374 aa). The soluble CADM1 band (72 kDa) and the recombinant soluble form of CADM1 were clearly detected in the sera of five patients with acute-type ATLL but not in the sera of five healthy volunteers (Figure 4a). We screened the sera of 5 healthy controls and 25 ATLL patients (14 acute-type, 7 lymphoma-type, 2 smoldering-type and 2 HTLV-1 carrier) for the presence of soluble CADM1. We detected different levels of soluble CADM1 among these ATLL patients by western blot (data not shown). In addition, we compared the levels of soluble CADM1 in the serum and the percentages of $CD4^+CADM1^+$ cells in the peripheral blood (Supplementary Figure 4) and confirmed that high levels of soluble CADM1 are present in the serum of patients who had high numbers of $CADM1^+$ cells in the peripheral blood. As serum levels of soluble IL-2Rα are correlated with the prognosis

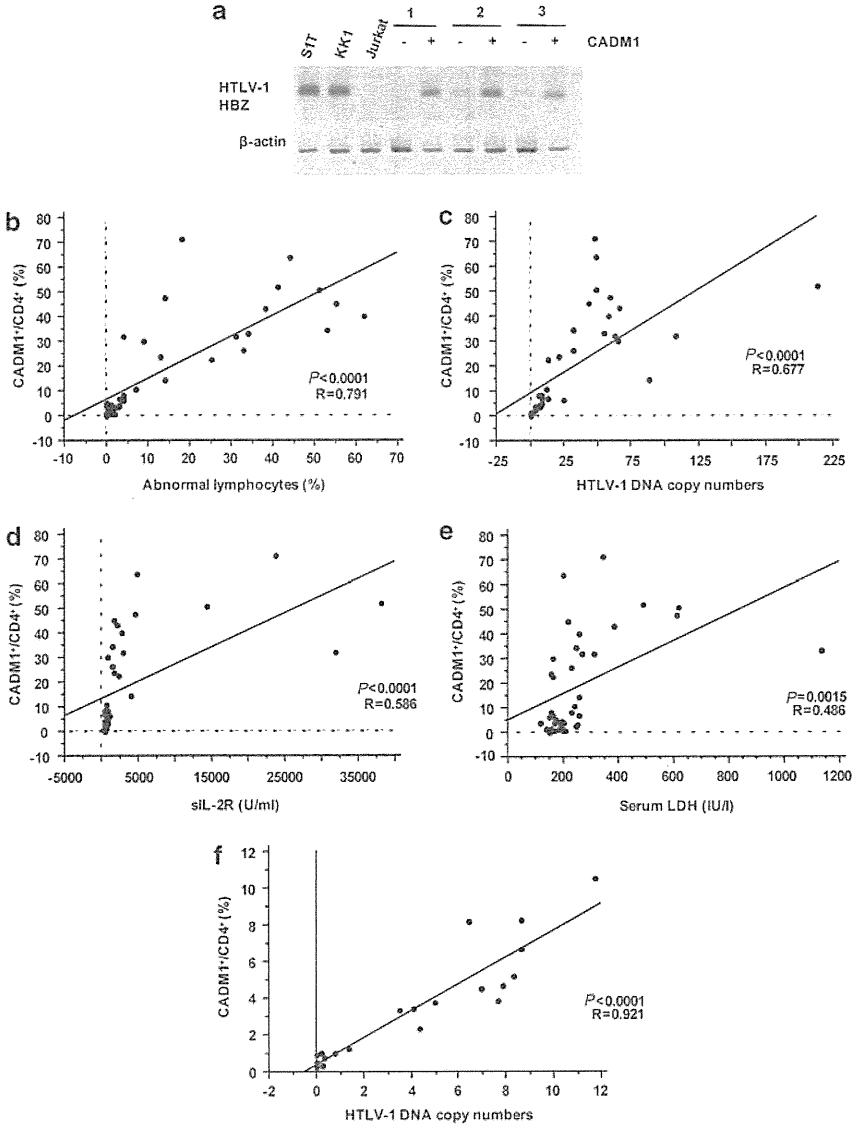

Figure 3. Correlation of the percentages of the $CD4^+CADM1^+$ fraction with the percentages of abnormal lymphocytes, HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. (**a**) Identification of the HTLV-1 genome by PCR amplification after separation by CADM1-magnetic beads. After separation of the peripheral blood of three ATLL patients by magnetic beads, genomic DNA was extracted from both the CADM1 and non-CADM1 fractions and amplified by specific PCR primers for HTLV-1 *HBZ*. Two ATLL cell lines (S1T and KK1) were used as positive controls, and a T-ALL cell line (Jurkat) was used as a negative control for the HTLV-1 *HBZ*. Lane 1, smoldering ATLL; lane 2, chronic ATLL; lane 3, HTLV-1 carrier. (**b-e**) The percentage of the $CD4^+CADM1^+$ fraction was compared with the percentage of abnormal lymphocytes, the HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. In (**d**), data from one acute-type patient were not included in the analysis because of the extremely high levels of soluble IL-2Ra ($CD4^+CADM1^+$, 32.9%; IL-2Ra, 96 900 U/ml). (**f**) The percentage of the $CD4^+CADM1^+$ fraction was compared with the HTLV-1 DNA copy number in HTLV-1 carriers.

of ATLL patients, we compared the serum levels of soluble CADM1 and soluble IL-2Rα in individual cases. As shown in Figure 4b, significantly higher levels of soluble CADM1 were detected in the serum of ATLL patients who had increased levels of soluble IL-2Rα; thus, serum CADM1 levels may be a diagnostic tool for the prediction of disease progression in ATLL.

High expression of CADM1 in ATLL-derived lymphomas

To examine the expression of CADM1 in tissue sections from lymphoma-type ATLL, formalin-fixed lymphoma samples from different types of malignant lymphomas were immunostained with the anti-CADM1 antibody. For these studies, we used a monoclonal antibody (1-10C) raised against the recombinant extracellular domain of the CADM1 protein. To confirm the reactivity of the anti-CADM1 antibody in formalin-fixed ATLL cells, cell pellets from various leukemia cell lines were fixed in 10% formalin, embedded in paraffin and stained for CADM1. The anti-CADM1 antibody specifically stained the surface of the CADM1-positive S1T-ATLL cell line but did not react with the CADM1-negative ED-ATLL and all non-ATLL cell lines (Figure 5a, panels 1 and 2, and Supplementary Figure 5a). Western blot analysis confirmed the lack of CADM1 expression in these cell lines (Figure 1a and Supplementary Figure 5b). We next performed immunostaining of lymph node biopsies from ATLL patients with malignant lymphoma using the anti-CADM1 antibody. As positive controls, we used erythrocytes and peripheral nerve tissue (Figure 5a, panels 3 and 4).[17,18] In addition, we examined CADM1

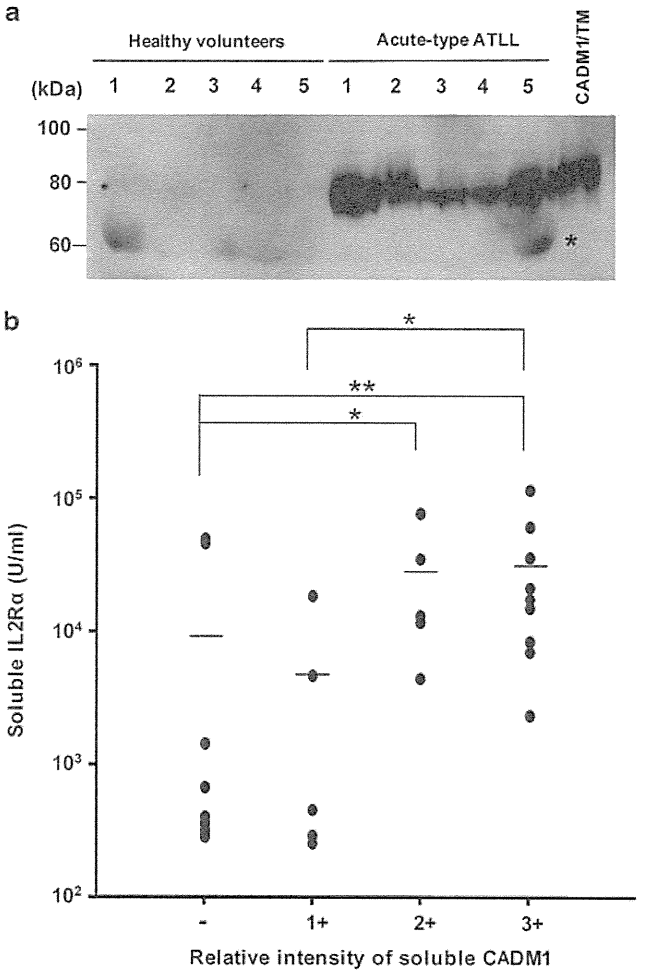

Figure 4. Identification of a soluble form of CADM1 in ATLL patients. (**a**) The soluble form of CADM1 in the peripheral blood from five healthy volunteers and five patients with acute-type ATLL was identified by immunoblot analysis using an anti-CADM1 antibody. The asterisk indicates an albumin band. Truncated CADM1 with an extracellular domain was purified from the culture supernatant of 293 cells after transfection of the CADM1 expression plasmid as a positive control. (**b**) The relative band intensity of CADM1 by immunoblot was compared with the level of sIL-2Rα in various serum samples from healthy volunteers, HTLV-1 carriers and ATLL patients. The band intensity was measured by the Image Gauge software (Fujifilm, Tokyo, Japan). The signal intensities were classified as either high (3+), medium (2+), low (1+) or undetectable (-). Asterisks indicate a significant difference between the band intensities of the groups (*$P<0.001$, **$P<0.0001$).

expression in three cases of lymph nodes with reactive follicular and/or paracortical hyperplasia (reactive lymph nodes) and found that most of the lymphocytes in the reactive lymph nodes were negatively stained and <1% of the cells were positively stained (Figure 5a, panel 5). The staining pattern of the CADM1-positive cells in the reactive lymph nodes mainly shows a uniform cytoplasmic pattern rather than the specific membranous staining that was seen in ATLL cells (as shown below and in Figure 1b). The CADM1-positive cells in reactive lymph node possibly correspond to histiocytes, including dendritic cells because a subset of T-cell zone dendritic cells was reported to express CADM1 (Necl-2) within the lymph node.[24,25] We examined 90 tissue samples from patients with various types of lymphoma, including 36 patients with ATLL and 54 with non-ATLL lymphomas, using erythrocytes and nerve fascicles as positive controls. Of the non-ATLL samples, 29 cases were T- or NK-cell lymphomas, 37 cases were B-cell lymphomas and 2 cases were null-cell lymphomas. Using a four-grade scale to score CADM1 immunohistochemical staining (0 to 3+, Figure 5b), we found that 92% of ATLL lymphomas were positive for CADM1, and 50% of them were heavily stained and were scored 2+ or higher (Table 1). Of note, a few lymphoma cells showed diffuse cytoplasmic staining in addition to membrane staining with CADM1. Among the non-ATLL lymphomas, a few CADM1-positive cells were observed, the morphology of which was small to medium in size with normochromatic round to ovoid nuclei and lacking nuclear atypia (Figure 5c). Based on the morphology and the CADM1-staining patterns, the CADM1-positive cells in the non-ATLL lymphomas were not considered as lymphoma cells but may correspond to histiocytes, including dendritic cells, because these cells were similar to the CADM1-positive cells found in reactive lymph nodes (Figure 5a, panel 5 and Figure 5c). Based on these results, a high degree of cell membrane staining for CADM1 with a score of 2+ may provide high specificity in the diagnosis of ATLL, and combined staining with CADM1 and other T-cell-specific markers may be necessary for a more accurate diagnosis of lymphoma-type ATLL.

DISCUSSION

In this study, we made a series of antibodies against CADM1 to be used as diagnostic tools for ATLL, such as for the identification and separation of ATLL and HTLV-1-infected cells, the detection of the soluble form of CADM1 in peripheral blood and the pathological identification of lymphoma-type ATLL after formalin fixation. Expression of CADM1 by flow cytometry was clearly detected on the surface of ATLL cells and HTLV-1-infected T lymphocytes, which was confirmed by detection of the HTLV-1 genome after separation by magnetic beads with a CADM1 antibody. The percentage of $CD4^+CADM1^+$ cells in the peripheral blood correlated highly with the DNA copy number of HTLV-1 in lymphocytes from HTLV-1 carriers and ATLL patients. In particular, we identified the soluble form of the CADM1 protein in the peripheral blood of HTLV-1 carriers and ATLL patients. The definitive diagnosis of ATLL is based on the confirmation of ATLL cells in the peripheral blood or in lymphoma tumors by detection of HTLV-1 genomic integration; therefore, measurement of serum levels of soluble CADM1 protein as well as detection of $CD4^+CADM1^+$ cells in the blood, when used in conjunction with other standard diagnostic methods, would be useful for identifying and monitoring disease progression in HTLV-1 carriers with increased accuracy and may aid in the early diagnosis and measurement of treatment effects for ATLL.

It has been proposed that HTLV-1 infects various types of cells, including T-reg cells and subsets of T helper cells (Th2 and Th17), in a cell-to-cell manner.[26-29] There is also evidence that ATLL cells act as T-reg cells that express CD4, CD25 and FoxP3 and are thought to contribute to the immune suppression of ATLL patients;[6] however, it was reported that CADM1 is expressed at low levels on resting naive T cells, and its expression is further downregulated 14 h following TCR activation.[30] Therefore, we determined the expression of CADM1 in the T-reg cell fraction of the peripheral blood of healthy volunteers. The results showed that a subset of the T-reg fraction weakly expressed CADM1, suggesting that CADM1 is not a major marker for the T-reg fraction and that CADM1 expression on ATL cells may reflect the fact that ATL cells originate from T-reg cells. As ATLL cells that constitutively express CD25 exhibited heterogeneous Foxp3 expression patterns,[5] a part of ATLL is likely derived from $FoxP3^+$ T-reg cells. In another report, a population of $FoxP3^+$ cells distinct from ATLL cells was shown to have a regulatory function and was found to impair the cell-mediated immune response to HTLV-1 in patients with ATLL.[31] Although we do not know whether the population of T-reg cells with weak expression of CADM1 in the

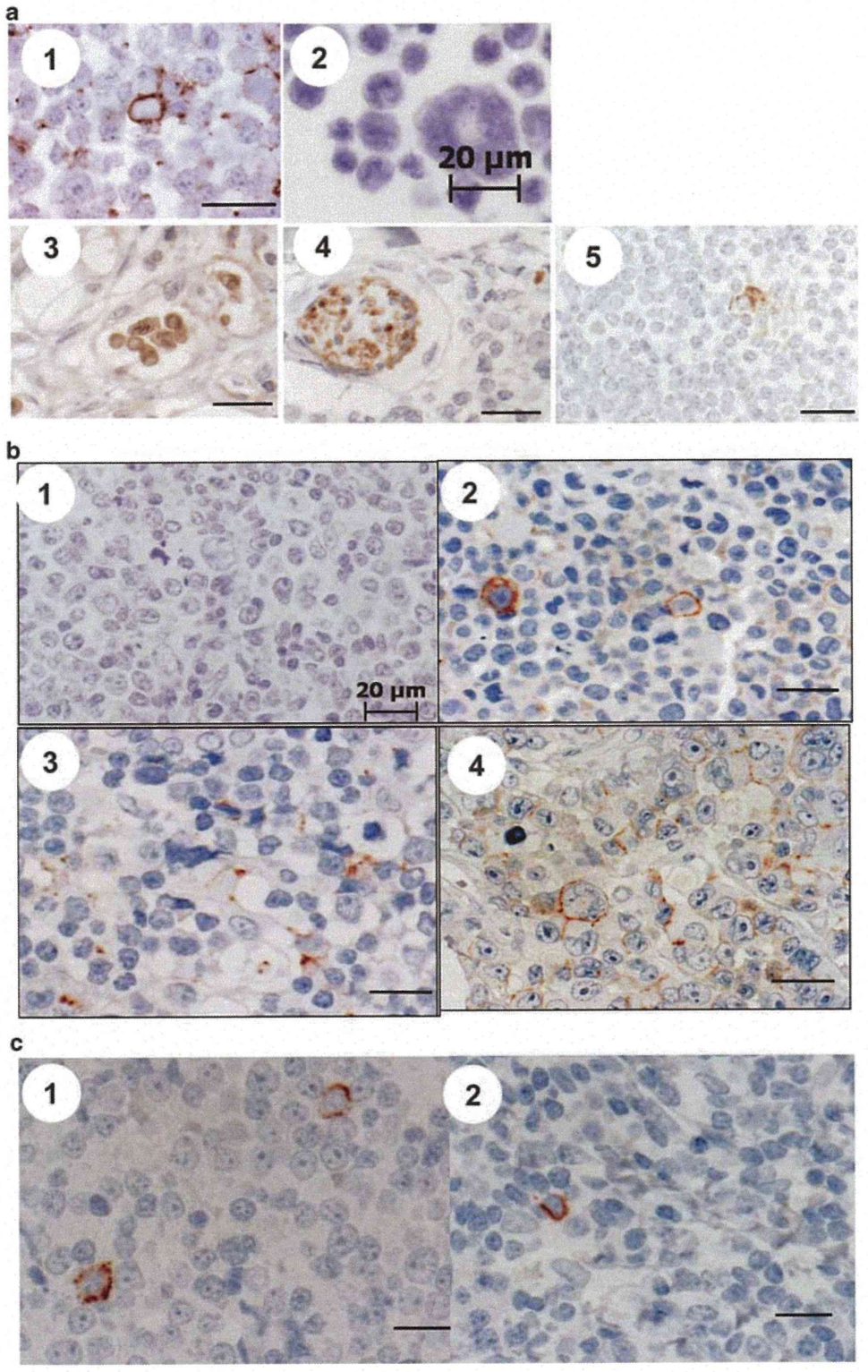

Figure 5. Expression of CADM1 in lymphoma-type ATLL. (**a**) Immunostaining of CADM1 in the S1T-ATLL cell line was used as a positive control (a1), and the ED-ATLL cell line was used as a negative control (a2) for CADM1 expression using an anti-CADM1 antibody (1–10C). Immunostaining of erythrocytes in the blood vessels (**c**), peripheral nerve cells (a3) and reactive lymph nodes (a4) using the same antibody. Scale bar, 20 μm. (**b**) The immunoreactivity for CADM1 was scored using a standardized four-tiered scoring scale as follows: staining in >30% of cells was scored as 3+ (b4); staining in >5% but <30% of cells was scored as 2+ (b3); staining in <5% of cells was scored as 1+ (b2); and a lack of staining was scored as 0 (b1). These images were taken from immunostained ATLL lymphoma sections. Scale bar, 20 μm. (**c**) Representative CADM1 immunostaining in B-cell (c1) and NK-cell (c2) lymphomas. Scale bar, 20 μm.

Table 1. Immunohistochemical staining of CADM1 in various types of lymphomas, including ATLL

	Case numbers	*Staining scores*				*Positive rates (%)*	
		Negative	*1+*	*2+*	*3+*	*⩾1+*	*⩾2+*
ATLL	36	3	15	14	4	33/36 (92)	18/36 (50)
Non-ATLL	54	37	16	1	0	17/54 (31)	1/54 (1.8)
T/NK	15	12	3	0	0	3/15 (20)	0/15 (0)
B	37	23	13	1	0	14/37 (38)	1/37 (2.7)
Null	2	2	0	0	0	0/2 (0)	0/2 (0)

Abbreviations: ATLL, adult T-cell leukemia/lymphoma; CADM1, cell adhesion molecule 1. The immunoreactivity for CADM1 was scored using a standardized four-tiered scoring scale as follows: staining in >30% of cells was scored as 3+; staining in >5% but <30% of cells was scored as 2+; staining in <5% of cells was scored as 1+; lack of staining was scored as 0.

PBMCs of healthy volunteers is the cellular origin for ATLL cells, CADM1 is thought to be one of the major markers for the various types of ATLL cells. In fact, we observed strong expression of CADM1 in rare cases of ATLL characterized by the $CD4^+CD8^+$, $CD4^-CD8^+$ or $CD4^-CD8^-$ phenotypes (data not shown); therefore, the $CADM1^{high}$ population of T-lymphocytes in peripheral blood can be considered ATLL cells.

The question of why CADM1 is strongly expressed on the surface of various types of ATLL remains unclear. Previously, we investigated whether the expression of CADM1 was induced by HTLV-1/Tax expression and found that Tax protein expression did not activate the expression of CADM1 in JPX-9 cells (data not shown). We also introduced a Tax expression vector into MOLT4 and 293T cells and determined the expression level of CADM1. We found that Tax could not induce CADM1 expression in these cells, suggesting that Tax expression is not related to the high expression of CADM1. As HBZ is known to be constitutively expressed in both HTLV-1-infected cells and ATLL cells and can modulate transcription of cellular genes,[16] it is possible that HBZ activates CADM1 expression. We also speculate that $CADM1^{high}$ expression in ATLL cells may be associated with transcriptional abnormalities in ATLL cells through the accumulation of genomic or epigenomic alterations. In this study, we found a good correlation between HTLV-1 copy numbers and the percentages of $CD4^+CADM1^+$ cells in the peripheral blood of HTLV-1 carriers, suggesting that HTLV-1 carriers with high percentages of $CD4^+CADM1^+$ cells could be associated with progressive genetic alterations and might be at high risk for developing ATLL.

Recent studies have shown that a few markers, such as CCR4 and CD70, are unique ATLL surface markers.[32,33] Although the proportion of $CD4^+CCR4^+$ cells and $CD4^+CD70^+$ cells in the PBMCs from healthy individuals were found to be approximately 5%,[27,33] the proportion of $CD4^+CADM1^+$ cells was <1% (Figure 2); therefore, measurement of $CADM1^+$ T cells is particularly efficient in the diagnosis of HTLV-1 infection in individuals who carry a small number of HTLV-1-infected cells. We have demonstrated previously that CADM1 has important functions in increasing cell adhesion and mediating progression to organ invasion.[19] In this study, we succeeded in isolating a low percentage of both HTLV-1-infected cells from the PBMCs of HTLV-1 carriers with high HTLV-1 copy numbers and ATLL cells from patients with ATLL. The sorted HTLV-1-infected cells and ATLL cells could become useful tools for transcriptional and/or genomic analysis that may be used to compare their results with those of PBMCs from either healthy volunteers or peripheral leukemia cells from patients with ATLL. The results may provide important information on the expression patterns and/or genomic abnormalities that occur at the early stages of HTLV-1 infection and/or ATLL development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)